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Simultaneous Liquid Chromatography Tandem Mass Spectrometric Determination of 35 Prohibited Substances in Equine Plasma for Doping Control

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Abstract : Many therapeutic class drugs such as beta-blocker, corticosteroids, NSAIDs, etc are prohibited substances in the horse racing industry. Liquid chromatography-tandem mass spectrometry (LC–MS/MS) technology makes it possible to isolate drugs from interference, enables various drug analyses in complex biological samples due to its sensitive sensitivity, and has been successfully applied to doping control. In this paper, we describe a rapid and sensitive method based on solid-phase extraction (SPE) using solid phase cartridge and LC–MS/MS to screen for different class's 35 drug targets in equine plasma. Plasma samples were pretreated by SPE with the NEXUS cartridge consisted non-polar carbon resin and minimum buffer solvent. Chromatographic separation of the analytes was performed on ACQUITY HSS C18 column (2.1 × 150 mm, 1.8 μm). The elution gradient was conducted with 5 mM ammonium formate (pH 3.0) in distilled water and 0.1% formic acid in acetonitrile at a flow rate of 0.25 mL/min. The selected reaction monitoring (SRM) mode was used for drug screening with multiple transitions in the positive ionization mode. The specificity, limit of detection, recovery, and stability was evaluated for validation. The method was found to be sensitive and reproducible for drug screening. The method was applied to plasma sample analysis for the proficiency test from the Association of Racing Chemist.

Keywords : Doping control, Equine plasma, LC–MS/MS

Introduction

Many therapeutic class drugs such as beta-blocker, corticosteroids, and NSAIDs, etc are prohibited substances in horse racing as specified in Article 6 of the International Agreement on Breeding, Racing and Wagering[1] published by the International Federation of Horseracing Authorities (IFHA) and in human sports in the World Anti-Doping Agency (WADA) Prohibited List,[2] respectively.

The screening of drugs in doping control is critical for sports fairness and horse welfare.[3-4] Drug testing methods have evolved over a long period. The method for the detection of drugs in biological samples before the 1970s started with the use of gas chromatography-mass spectrometry (GC-MS).[5] Currently, liquid chromatography-mass spectrometry (LC-MS) is the most widely used method for doping testing in sports. The LC-MS method allowed the analysis of thermally labile substances with a wide range of molecular weights faster and with better sensitivity.[6–8] A recent trend has emerged in doping control for animal and human sports, utilizing tandem mass spectrometry for drug detection due to significant technological improvements in selectivity, sensitivity, and robustness in tandem mass spectrometry.[9-12] The coupling of liquid chromatography and tandem mass spectrometry (LC-MS/MS) has also made it possible to screen drugs in complex biological fluids. In LC–MS/MS, mass filtering can separate the effective signals of the target drug from interferences to improve detection ability. Theoretically, most drugs of less than 1000 Da could be analyzed in a single analytical run.

In many years, the analysis of plasma has become increasingly popular for doping control purposes because sample collection is easier and has less analytical matrix effect and variation than urine, relatively. In blood analysis, the parent drug in blood can usually be used as an analysis target, but in urine, drug metabolites may be used as the main analysis target, so it is often difficult to obtain

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reference standards for analysis.[12] However, the blood concentration is very low, so the use of a sensitive instrument is essential.

In this paper, we describe a rapid and sensitive method based on solid-phase extraction (SPE) using a solid-phase cartridge consisting of non-polar carbon resin and LC–MS/MS to screen for different class's 35 drug targets in equine plasma. Method validation parameters including specificity, sensitivity, extraction recovery, and stability are evaluated. The method was applied to plasma sample analysis for the proficiency test from the Association of Racing Chemist (AORC).

Materials and methods

Materials

Acepromazine maleate, atenolol, amitriptyline hydrochloride, betamethasone, chlorpromazine hydrochloride, dexamethasone, diclofenac sodium, flunixin meglumine, ketamine, ketoprofen, lidocaine, meloxicam, mepivacaine hydrochloride, methylprednisolone, pentazocine, pseudoephedrine hydrochloride, reserpine, salbutamol, stanozolol and triamcinolone acetonide were purchased from USP (Rockville, MD, USA). Boldenone, nandrolone, and testosterone were purchased from Steraloids (Newport, RI, USA). Clenbuterol hydrochloride, ephedrine, firocoxib, flumethasone, fluphenazine hydrochloride, methocarbamol, nordiazepam, piroxicam, sildenafil citrate, terbutaline hemisulfate salt, phenacetin and ammonium formate were purchased from Sigma-Aldrich (St. Louis, MO, USA). Detomidine hydrochloride was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). HPLC-grade acetonitrile (ACN), HPLC-grade distilled water (DW), and methanol (MeOH) were purchased from J.T. Baker (Phillipsburg, NJ, USA). Formic acid (FA), hexane, and ammonium hydroxide (NH_4OH) were purchased from Junsei Chem (ChouKuu, Japan). ABS ELUT Nexus cartridges (60 mg/3 mL) were purchased from Agilent Technologies (Les Ulis, France).

Instrumentation

Chromatographic separation and ESI conditions were followed based on the previously published author's paper.[8] Chromatographic separation of the analytes was performed using the Sciex Exion UHPLC system on ACQUITY HSS C18 column (2.1 × 150 mm, 1.8 μm). The elution gradient was conducted with 5 mM ammonium formate (pH 3.0) in DW (mobile phase A) and 0.1% FA in acetonitrile (mobile phase B) at a flow rate of 0.25 mL/min, 10% mobile phase B for 1 min, 10–40% mobile phase B for 2 min, 40%–95% mobile phase B for 4.5 min, 95% mobile phase B for 0.5 min, 10% mobile phase B for 0.1 min, and 10% mobile phase B for 3.5 min. The injection volume was 5 μL. Tandem mass (MS/MS) analysis was performed on AB Sciex QTRAP 6500 (Toronto, Canada). The ionization was performed in the positive mode using the Turbo Ionspray source at a temperature of 550°C. The ion spray voltage was 5500 V. Selected reaction monitoring (SRM) for detection was used with 150 s set for the detection window. The transitions optimized for 35 analytes and ISTD were summarized in Table 1. Data processing and handling were performed by MultiQuant 3.0.2 and Analyst 1.6 software.

Preparation of stock and working solutions

The stock solutions of analytes and internal standard (ISTD) were independently prepared by weighing the suitable quantity of reference material at a concentration of 1000 μg/mL in MeOH. The working solutions were prepared by dilution of the stock solution with MeOH. The stock solutions and working solutions were kept at -20°C.

Sample preparation

The equine blood sample in a heparin tube was centrifuged for 10 minutes at 3000 rpm and the plasma supernatant was separated. A solid phase extraction was conducted on Nexus (3 mL, 60 mg) cartridge. The ISTD (10 μg/mL, 20 μL) was added to the plasma sample (2 mL). Then the plasma sample was adjusted to pH 9 or higher by adding 1 mL of 2% NH_4OH. The sample (3 mL) was loaded onto the cartridge. The cartridge was washed using 3 mL of DW and 3 mL of hexane, dried for 3 minutes, washed with MeOH (3 mL), and dried again for two minutes. Analytes were eluted using 3 mL of MeOH. The solvent was evaporated under nitrogen at 50°C. The residue was finally vortexed with LC initial solvent (50 μL) and transferred into a vial.

Method validation

The specificity, limit of detection (LOD), recovery, and stability were measured for analytical method validation.

Specificity

Specificity was tested by checking for possible interfering peaks in SRM for analytes and the ISTD from blank plasma samples of 20 different origins

LOD and recovery

LOD was estimated based on a signal-to-noise ratio of (S/N) at least three measured peak to peak (n = 10). The recovery of analytes was measured by spiking the standard solution of analytes at 50 ng/mL (n = 5).

Stability

Stability tests of analytes were performed at a concentration level of 50 ng/mL. The stability tests were conducted at 25°C for 1 day and 7 days and at -4°C for 1 day and 7 days. (n = 5 each).

Application

The developed method for each analyte was applied to plasma samples for the proficiency test from AORC.[13]

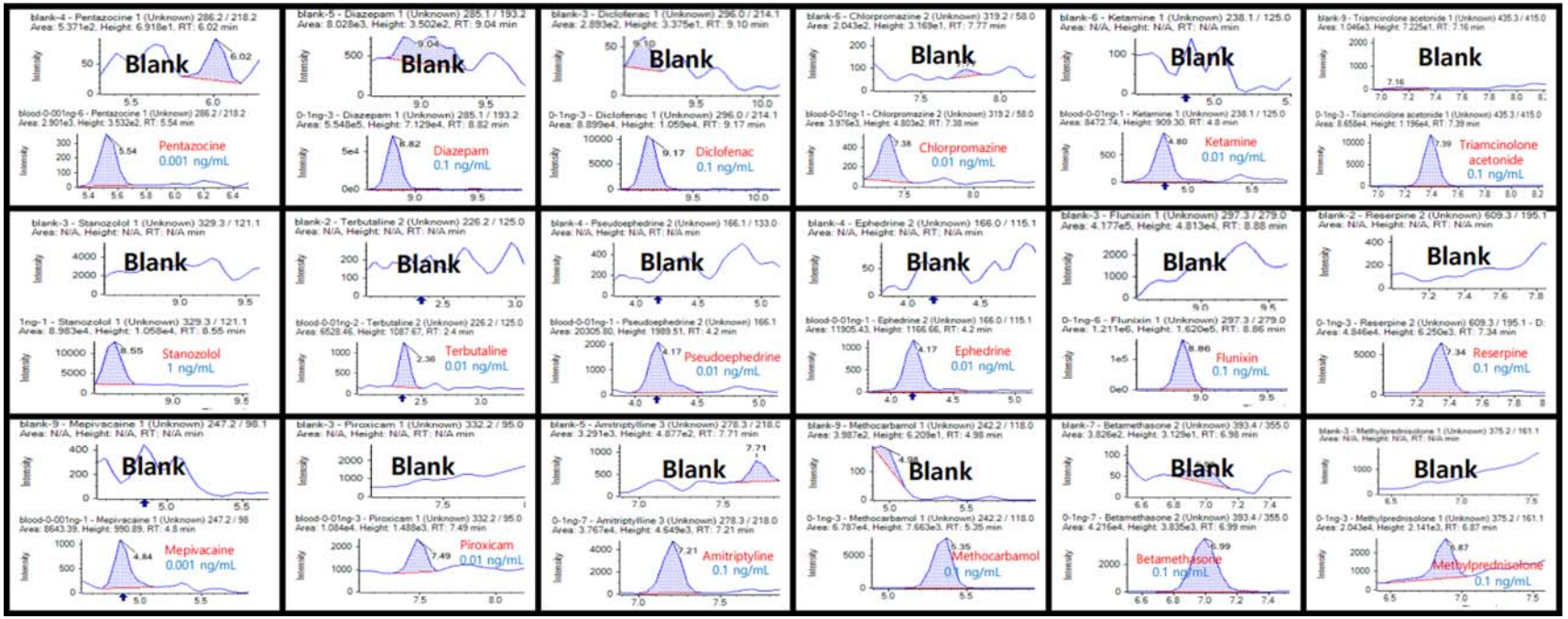

Figure 1. Blank chromatograms and the 35 target analytes' chromatograms obtained from a spiked equine plasma sample at LODs.

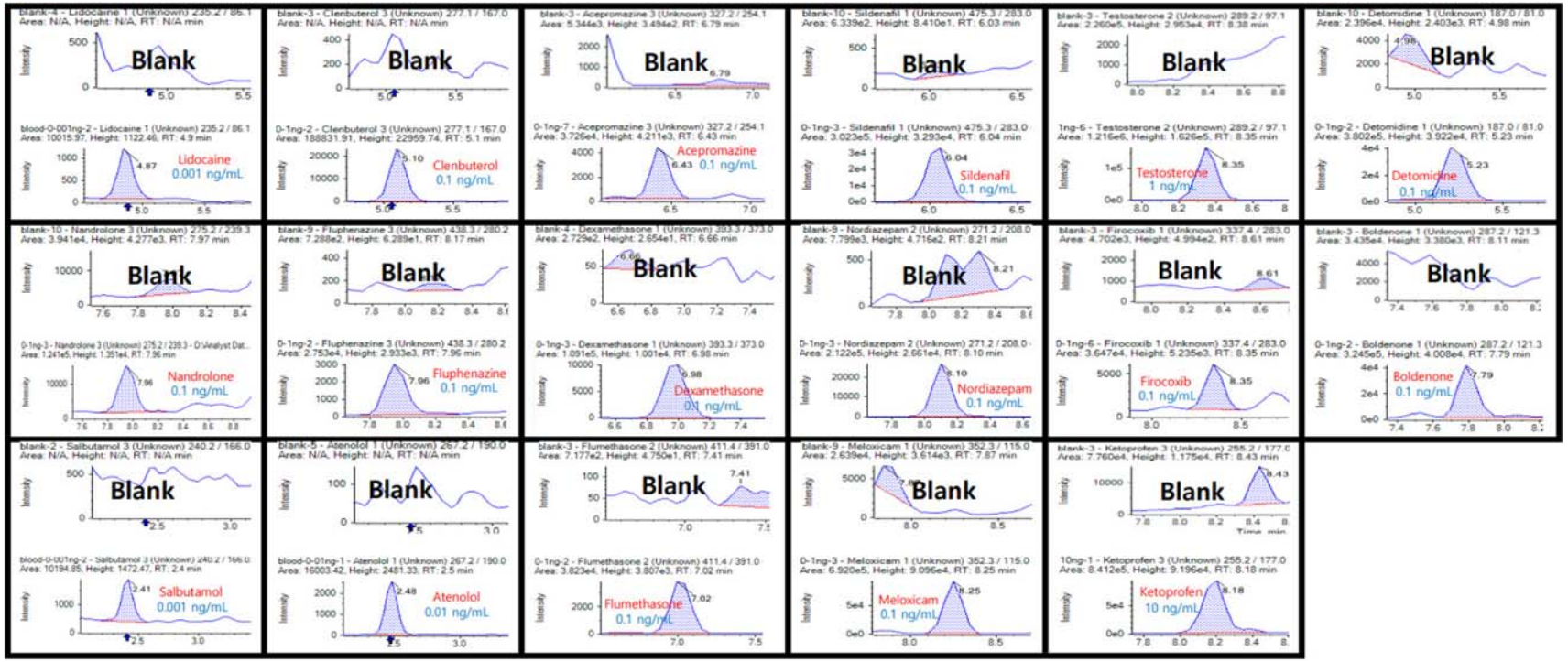

Figure 1. (continued)

Results and Discussion

Solid-phase extraction

The extraction procedure from plasma was simple and straightforward that based on a non-polar retention mechanism with no pre-conditioning required and minimum buffer solvent used to cover more than 30 analytes in different drug classes including tranquilizers, beta-blockers, corticosteroids, etc. The SPE protocol was the improved approach compared to previous WCX cartridge extraction which was complicated using many buffers to screen for varieties of drugs with LC–MS/MS in the author's laboratory. Plasma samples are fast and simply extracted using single solvents (DW, hexane, MeOH) in the process other than using a basic solvent to adjust the pH of the sample at the first step.

Chromatography and spectrometry

SRM conditions for analyte analysis were optimized by infusing reference standard solutions dissolved in MeOH directly into the mass spectrometer under declustering potential (DP), entrance potential (EP), collision energy (CE), and collision cell exit potential (CXP). All analytes stably produced protonated ions in the positive mode. All analytes which were performed by chromatographic separation on an HSS C18 column with acidic base LC mobile solvents showed good peak shapes. The elution time for analytes was within 9.5 min (Figure 1 and Table 1).

Specificity

The specificity was determined with different origin plasma samples (n = 20). The significant interference at the

Table 1. Mass spectrometry parameters and retention times for the 36 substances. The DP, EP, CE, and CXP were optimized for SRM transitions.

Name	Therapeutic classification	Polarity	Precursor ion (m/z)	Product ion (m/z)	DP (V)	EP (V)	CE (V)	CXP (V)	RT (min)
Acepromazine	Tranquilizer	+	327.2	254.1	80	10	35	11	6.43
				86.1	80	10	35	11	
Atenolol	β-Blocker	+	267.2	190.0	60	10	27	11	2.48
				145.0	60	10	40	11	
Amitriptyline	Antidepressant	+	278.3	218.0	60	10	35	11	7.21
				191	60	10	35	11	
Betamethasone	Corticosteroids	+	393.4	355.0	45	10	18	11	6.99
				373.0	45	10	13	11	
Boldenone	Anabolic steroid	+	287.2	121.3	65	10	35	11	7.79
				135.0	65	10	20	11	
Chlorpromazine	Tranquilizer	+	319.2	58.0	60	10	55	11	7.38
				86.0	60	10	30	11	
Clenbuterol	Bronchodilator	+	277.1	167.0	40	10	35	11	5.10
				259.0	40	10	17	11	
Detomidine	Sedative	+	187.0	81.0	81	10	29	11	5.23
Dexamethasone	Corticosteroids	+	393.3	373.0	45	10	13	11	6.98
				355.0	45	10	18	11	
Diazepam	Anxiolytic	+	285.1	193.2	100	10	44	11	8.82
				154.1	100	10	38	11	
Diclofenac	NSAID	+	296.0	214.1	80	10	50	11	9.17
				250.0	80	10	20	11	
Ephedrine	Sympathomimetic	+	166.0	115.1	70	10	36	11	4.17
				148.0	70	10	15	11	
Firocoxib	NSAID	+	337.4	283.0	65	10	14	11	8.35
				237.0	65	10	21	11	
Flunixin	NSAID	+	297.3	279.0	75	10	32	11	8.86
				264.0	75	10	45	11	
Flumethasone	Corticosteroids	+	411.4	391.0	50	10	13	11	7.02
				371.0	50	10	16	11	

Table 1. Continued.

Name	Therapeutic classification	Polarity	Precursor ion (*m/z*)	Product ion (*m/z*)	DP (V)	EP (V)	CE (V)	CXP (V)	RT (min)
Fluphenazine	Antipsychotic	+	438.3	280.2	20	10	40	11	7.96
				171.1	20	10	34	11	
Ketamine	Anesthetic	+	238.1	125.0	75	10	46	11	4.80
				220.2	75	10	20	11	
Ketoprofen	NSAID	+	255.2	177.0	70	10	26	11	8.18
				209.0	70	10	21	11	
Lidocaine	Local analgesic	+	235.2	86.1	100	10	30	11	4.87
				58.0	100	10	51	11	
Meloxicam	NSAID	+	352.3	115.0	55	10	25	11	8.25
				141.0	55	10	21	11	
Mepivacaine	Local anesthesic	+	247.2	98.0	80	10	35	11	4.84
				70.0	80	10	50	11	
Methocarbamol	Skeletal muscle relaxant	+	424.2	118.0	55	10	14	11	5.35
				199.0	55	10	13	11	
Methylprednisolone	Corticosteroids	+	375.2	161.1	60	10	23	11	6.87
				185.4	60	10	30	11	
Nandrolone	Anabolic steroid	+	275.2	239.3	80	10	20	11	7.96
				257.3	80	10	20	11	
Nordiazepam	Anxiolytic	+	271.2	208.0	70	10	40	11	8.10
				140.0	70	10	40	11	
Pentazocine	Opioid analgesic	+	286.2	218.2	80	10	27	11	5.54
				173.2	80	10	38	11	
Piroxicam	NSAID	+	332.2	95.0	50	10	22	11	7.49
				121.0	50	10	29	11	
Pseudoephedrine	Sympathomimetic	+	166.1	148.0	30	10	15	11	4.17
				133.0	30	10	28	11	
Reserpine	Antihypertensive	+	609.3	195.1	100	10	38	11	7.34
				397.1	100	10	47	11	
Salbutamol	β -agonists	+	240.2	166.0	35	10	20	11	2.41
				222.0	35	10	15	11	
Sildenafil	Vasodilator	+	475.3	283.0	90	10	52	11	6.04
				311.0	90	10	40	11	
Stanozolol	Anabolic steroids	+	329.3	121.1	60	10	45	11	8.55
				95.1	60	10	45	11	
Terbutaline	Bronchodilator	+	226.2	125.0	50	10	33	11	2.36
				152.0	50	10	22	11	
Testosterone	Anabolic steroid	+	289.2	97.1	80	10	35	11	8.35
				109.1	80	10	35	11	
Triamcinolone acetonide	Corticosteroids	+	435.3	415.0	50	10	15	11	7.39
				339.0	50	10	20	11	
Phenacetine (ISTD)	-	+	180.0	110.0	80	10	28	11	6.05

retention times of the transitions was not observed. A suspicious sample could be further evaluated through follow-up analysis using multiple SRMs (Table 1).

LOD and recovery

All target analytes were consistently detectable in spiked samples. The LODs listed in Figure 1 and Table 2 were measured by the lowest concentrations evaluated at an S/N ratio of greater than 3:1 in the SRM chromatogram. The LODs for the different analytes were between 0.001 and 10 ng/mL with over 31% of the analytes having LODs at or below 0.01 ng/mL, and over 92% at or below 0.1 ng/mL. The LOD of ketoprofen was measured relatively high at 10 ng/mL. These results show the improved sensitivity for 21 analytes (atenolol, amitriptyline, boldenone, dexamethasone, diazepam, firocoxib, flumethasone, fluphenazine, ketamine, lidocaine, mepivacaine, methylprednisolone, nandrolone, nordiazepam, piroxicam, pseudoephedrine, reserpine,

Table 2. Method validation results: limit of detection (LOD), recovery, and stability.

Name	LOD (ng/mL) (n = 10)	Recovery (%) (n = 5)	Stability (%) (n = 5)			
			25°C		-4°C	
			1day	7day	1day	7day
Acepromazine	0.1	56.9±14.1	107.7	112.8	108.0	105.3
Atenolol	0.01	83.8±5.2	102.0	100.3	107.5	105.3
Amitriptyline	0.1	64.7±16.2	104.3	105.2	100.7	140.9
Betamethasone	0.1	95.4±16.1	91.6	103.8	97.2	100.2
Boldenone	0.1	75.4±11.6	101.2	103.1	98.5	102.1
Chlorpromazine	0.01	44.4±14.2	106.4	105.7	99.9	106.4
Clenbuterol	0.1	84.9±8.1	101.5	108.4	106.3	98.5
Detomidine	0.1	78.6±17.3	104.9	107.7	107.1	103.5
Dexamethasone	0.1	91.0±14.8	106.1	110.8	108.5	103.8
Diazepam	0.1	50.7±7.3	101.0	108.2	109.1	95.3
Diclofenac	0.1	21.3±11.5	100.6	99.4	101.2	97.8
Ephedrine	0.01	55.4±3.7	101.0	103.1	105.5	106.6
Firocoxib	0.1	74.6±7.6	100.2	95.5	97.8	100.6
Flunixin	0.1	31.1±4.6	102.2	103.2	99.9	103.9
Flumethasone	0.1	85.1±14.2	105.9	107.3	104.7	107.3
Fluphenazine	0.1	18.4±14.9	107.7	110.0	101.0	105.3
Ketamine	0.01	70.6±9.0	104.0	98.0	102.0	102.5
Ketoprofen	10	23.4±7.3	92.5	110.6	103.9	106.4
Lidocaine	0.001	81.0±8.2	98.7	101.9	104.1	109.5
Meloxicam	0.1	46.8±4.9	109.0	102.2	105.9	98.9
Mepivacaine	0.001	100.4±6.4	97.4	100.3	106.3	103.0
Methocarbamol	0.1	88.9±14.1	103.3	100.4	107.1	103.2
Methylprednisolone	0.1	74.6±11.6	109.5	107.7	104.7	108.2
Nandrolone	0.1	62.7±9.9	100.2	98.5	99.8	99.7
Nordiazepam	0.1	69.4±9.8	101.2	104.6	109.1	108.0
Pentazocine	0.001	68.0±14.4	102.5	100.8	104.6	104.4
Piroxicam	0.01	49.3±6.9	100.9	104.0	109.1	98.6
Pseudoephedrine	0.01	56.3±3.7	93.0	102.0	101.6	107.0
Reserpine	0.1	20.5±10.3	103.7	103.2	103.9	94.1
Salbutamol	0.001	50.4±3.0	108.1	101.1	105.3	109.8
Sildenafil	0.1	77.4±8.4	99.5	100.5	100.4	97.5
Stanozolol	1	59.0±15.0	97.5	98.4	100.8	100.1
Terbutaline	0.01	44.9±3.2	105.1	109.2	97.1	107.5
Testosterone	1	68.9±7.0	100.2	99.4	97.4	98.6
Triamcinolone acetonide	0.1	85.1±14.3	106.6	108.6	103.2	105.2

Table 3. Drug analysis results in proficiency plasma drug test from AORC. The minimum concentrations spiked in blank plasma for the proficiency test were acepromazine (1 ng/mL), dexamethasone (0.2 ng/mL), methylprednisolone (0.1 ng/mL), and testosterone (0.5 ng/mL), respectively.

Sample number	Detected analytes
Plasma 16-A	Dexamethasone, methylprednisolone
Plasma 16-B	Blank
Plasma 16-C	Acepromazine, testosterone

salbutamol, sildenafil, terbutaline, triamcinolone acetonide) in a short LC run time compared to the previous method.[12] The mean extraction recovery of analytes was between 18.4% to 100.4% with a maximum RSD of 17.3%. The result means that most of the analytes were well recovered from plasma after SPE with the nexus cartridge. The analytes with low recoveries below 50 % showed sensitive LODs below 10 ng/mL (Table 2).

Stability

Spiked plasma samples were stable for 7 days at 25°C and -4°C. These results mean that the analytes are stable during all analytical procedures. The data obtained are shown in Table 2.

Application

The developed method was applied to the analysis of plasma samples with a proficient plasma drug test from AORC. Three plasma test samples were analyzed, one sample was blank, and dexamethasone, methylprednisolone, acepromazine, flufenamic acid, and testosterone were detected in other two samples (Table 3).

Conclusion

In conclusion, a sensitive, rapid, and simple method for the analysis of 35 prohibited drugs in equine plasma was developed. Significant interference from the matrices was not observed. The LODs for the plasma method ranged from 0.001 ng/mL to 10 ng/mL and the mean recovery was between 18.4% to 100.4% with a maximum RSD of 17.3%. The plasma samples were stable for 7 days at 25°C and-4°C. The method was successfully applied to plasma sample analysis for the proficiency test from AORC.

Conflicts of interest

The authors declare no conflict of interest.

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